Biochimica et Biophysica Acta, 777 (1984) 140–146
Elsevier

BBA 72281

AN X-RAY DIFFRACTION AND ELECTRON MICROSCOPY STUDY OF THE EXTRACTION OF ERYTHROCYTE MEMBRANES WITH THE BILE SALT, CHOLATE

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(Received May 23rd 1984)

Key words: Erythrocyte membrane; Cholate extraction; X-ray diffraction; Freeze-fracture; Cytoskeleton; Cryopreservation

Studies by X-ray diffraction and electron microscopy of slowly frozen samples of control and cholate-extracted preparations of erythrocyte membranes have demonstrated changes in structural parameters which can readily be related to the extraction of cytoskeletal proteins. In the frozen state, these components appear to be condensed to about 10% of the total membrane thickness. The observations illustrate some of the advantages and limitations in the use of slowly frozen membrane preparations in studies of membrane structure.

Introduction

Comparisons of structural parameters of hydrated membranes by X-ray diffraction have been limited by the problem of maintaining a constant low level of hydration of the sample during the recording of diffraction patterns. We have reported previously that such conditions can be established by maintaining membrane preparations in the frozen state during diffraction exposures [1]. The rationale of this approach is to withdraw excess water from the aqueous interfaces in membrane stacks by the formation of pockets of ice crystals during slow freezing. This procedure is equivalent to that used in the cryopreservation of tissues [2] in which an initial slow freezing induces extracellular ice formation and consequent partial dehydration of the cytosol, so that in the subsequent rapid freezing to liquid nitrogen temperatures, intracellular ice formation is minimised. As this two-step freezing process is effective in the preservation of cell viability, it can be inferred that it causes minimum damage to membrane structure. The effect of slow freezing on membrane stacks is to reduce dramatically the separation of the membranes and to improve their alignment. At a constant freezing temperature, the level of hydration in the slowly frozen membrane stacks is maintained at a constant low level and the definition and intensities of diffraction patterns are substantially improved. Rapid or ultra-rapid freezing prevents water migration and thereby maintains the large and somewhat irregular periodicity of the initial membrane preparation, so that diffraction is weak and ill-defined.

This paper reports the use of the slow-freezing approach in a study of partial extraction of erythrocyte membranes by treatment with the trihydroxy bile salt, cholate. We showed previously [3] that this detergent extracts from this membrane preparation those proteins which form the cytoskeletal meshwork [4,5].

Materials and Methods

Haemoglobin-free erythrocyte ghosts were prepared from human blood (4–7-day-old samples from the Midland Blood Transfusion Centre) by haemolysis and repeated washing with 10 vol 20 imosM Tris buffer (pH 7.2)/1 mM EDTA [1,3].

Samples of loosely packed membranes separated by low speed (16000 × *g* for 15 min) centrifugation were dispersed in 10 vol. 0.5% or 1.5% cholate (Sigma, London, U.K.) in Tris buffer (pH 7.2) and incubated at 4 or 37°C for 30 min. The residual membranes were separated at 16000 × *g* for 15 min and washed twice by resuspension in 10 vol. of the standard buffer before being deposited as a very tightly packed layer, 0.5–1 mm thick, on a disc of thin plastic (Melinex) sheet by centrifugation under isopotential conditions [6] provided by specially constructed inserts for swing-out ultracentrifuge tubes. Centrifugation was at 120000 × *g* or at 200000 × *g* for 16–20 h at 4°C.

X-ray diffraction

The disc carrying the layer of tightly packed membranes was clamped (at the edges) over a stainless-steel rod inside a vacuum-tight chamber in an X-ray diffraction camera. This chamber was of small internal dimensions but was part of a relatively massive brass block, the temperature of which was controlled thermoelectrically. Temperatures between −40 and +50°C could be maintained to better than ±1/2 K in the evacuated diffraction camera. Sample temperatures were estimated by reference to diffraction from a series of pure chemical compounds with melting points covering the required range of low temperatures. Relatively slow cooling rates (up to 18 K/min) were used in these experiments.

Evacuated diffraction cameras incorporating mirror-focussing of X-rays [1] were used in conjunction with an Elliott GX6 rotating anode X-ray generator. Samples were aligned with the X-ray beam by raising the supporting stainless-steel rod until the beam intensity was reduced by 40–50%.

Electron microscopy

Thin sections. A small segment of each sample disc was taken for electron microscopy immediately prior to loading the remainder into the diffraction camera. Such samples were fixed immediately in buffered osmium tetroxide and processed by standard procedures for thin sectioning. Samples which had been frozen for recording X-ray diffraction patterns were also subsequently thawed and processed for electron microscopy.

Freeze-fracture. In order to obtain consistently high quality freeze-fracture replicas, glycerol (up to 20%) was generally introduced into the dispersed membrane preparation before the final centrifugation. Some studies were undertaken in which cryoprotectant was not used, but these provided only qualitative support to the freeze-fracture observations. Samples for freeze-fracture were taken either immediately after centrifugation or after subsequent partial dehydration under controlled humidity conditions. Some samples were frozen rapidly to −150°C and others were frozen relatively slowly (18 K/min) to −30°C to mimic diffraction conditions and then rapidly to −150°C for freeze-fracture. The frozen samples were accommodated in a sandwich-type holder which was broken open by a movement of the specimen 'shroud' surrounding the cold stage in a modified Edwards Freeze-Fracture unit. The replicas were examined on a tilt stage in the Philips 301 electron microscope.

Chemical analysis

Supernatants and residual membranes from cholate extraction experiments were analysed for phospholipid and protein, and the polypeptides were identified by sodium dodecyl sulphate polyacrylamide gel analysis after lipid extraction and trichloroacetic acid precipitation [3].

Results

Treatment of erythrocyte membranes with 0.5% cholate at 4 or 37°C, or with 1.5% cholate at 4°C, achieved negligible (less than 2%) protein extraction and no changes in structural parameters were detected either by X-ray diffraction or by electron microscopy. The membranes had not vesiculated. These preparations served as controls for comparison with cholate-extracted samples.

Significant protein extraction (40%) was consistently achieved with 1.5% cholate at 37°C for 30 min. Under these conditions, SDS-polyacrylamide gel analysis showed a preferential and substantial extraction of polypeptides corresponding to bands 1/2, 2.1, 4.1, 5 and 6. The gel profiles were not significantly different from those published previously [3]. 60–70% of membrane phospholipid was also extracted under these conditions. Electron micrographs of lightly centrifuged (16000 × *g* for

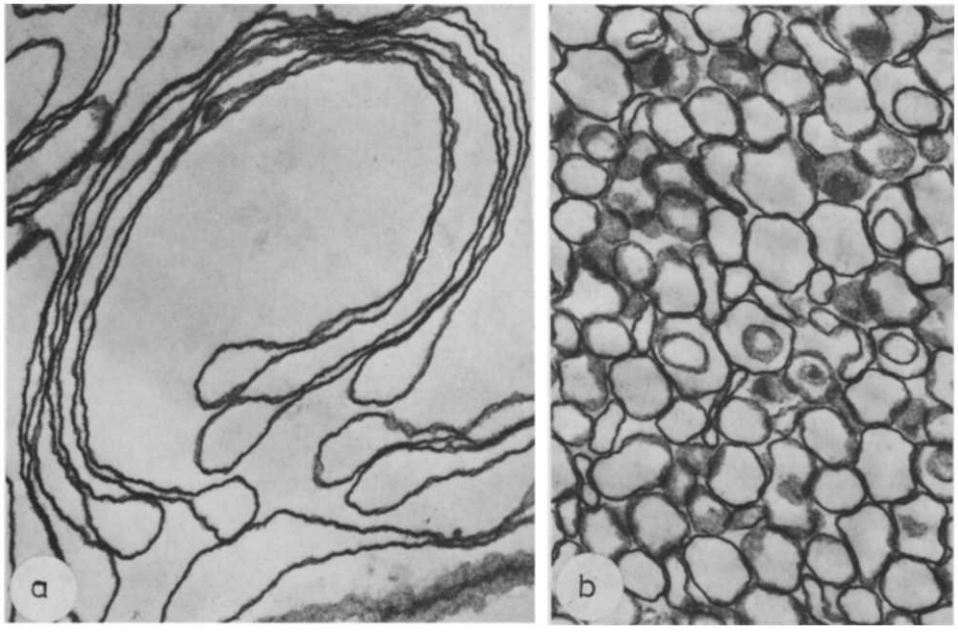


Fig. 1. Electron micrographs of thin sections of control (a) and cholate-extracted (b) erythrocyte ghosts. Samples had been centrifuged at low speed (16000× *g* for 15 min) before fixation. Magnification ×26000.

15 min) samples (Fig. 1) showed a disruption of the ghosts to give 0.5–1 μm diameter vesicles. The trilaminar unit of the membrane was more clearly defined than in non-extracted membranes.

In samples centrifuged on to 'Melinex' discs for X-ray diffraction, the vesicles had formed collapsed sacs (Fig. 2b), but these remained loosely packed as compared with the intact ghosts in control samples (Fig. 2a). A closer packing of cholate-modified membranes was observed in electron micrographs of samples fixed following diffraction in the frozen state and in samples that had been partially dehydrated before fixation. A decrease in the magnitude of the cytoplasmic separation of membrane trilaminar units relative to the external separation was clearly indicated in these close-packed arrays of membranes.

The presence of glycerol in the modified membrane preparation (as used for freeze-fracture electron microscopy) reduced the extent of collapse of vesicles as seen both in sections and in freeze-fracture replicas so that in the presence of 20% glycerol, no significant condensation of the vesicular system was achieved even by prolonged centrifugation at the highest available *g* forces. This dispersion of the membrane vesicles was maintained when fast freezing was employed in the preparation of freeze-fracture replicas (Fig. 2d) and no 'stepped' system of membranes could be observed. However, an initial slow-freezing step did induce a greater collapse of vesicles and closer packing of membranes (Fig. 3c, d, e). In such preparations, multiple steps were occasionally observed which clearly showed that in cholate-treated preparations, alternate steps were quite similar, whereas in control samples, (Figs. 2c and 3a) they were markedly different. In fractures through closely packed, flattened ghosts or vesicles, steps between successive fracture faces represent two half-membranes plus an intermembrane separation which is alternately that between cytoplasmic surfaces (the cytoplasmic separation) and that between external surfaces (the external separation). The inference from the micrographs is therefore that the thickness of the cytoplasmic separation has been reduced substantially by cholate extraction. There was no indication in any of the electron micrographs of any more extensive reorganisation of membrane structure.

Comparison of freeze-fracture replicas of fast-

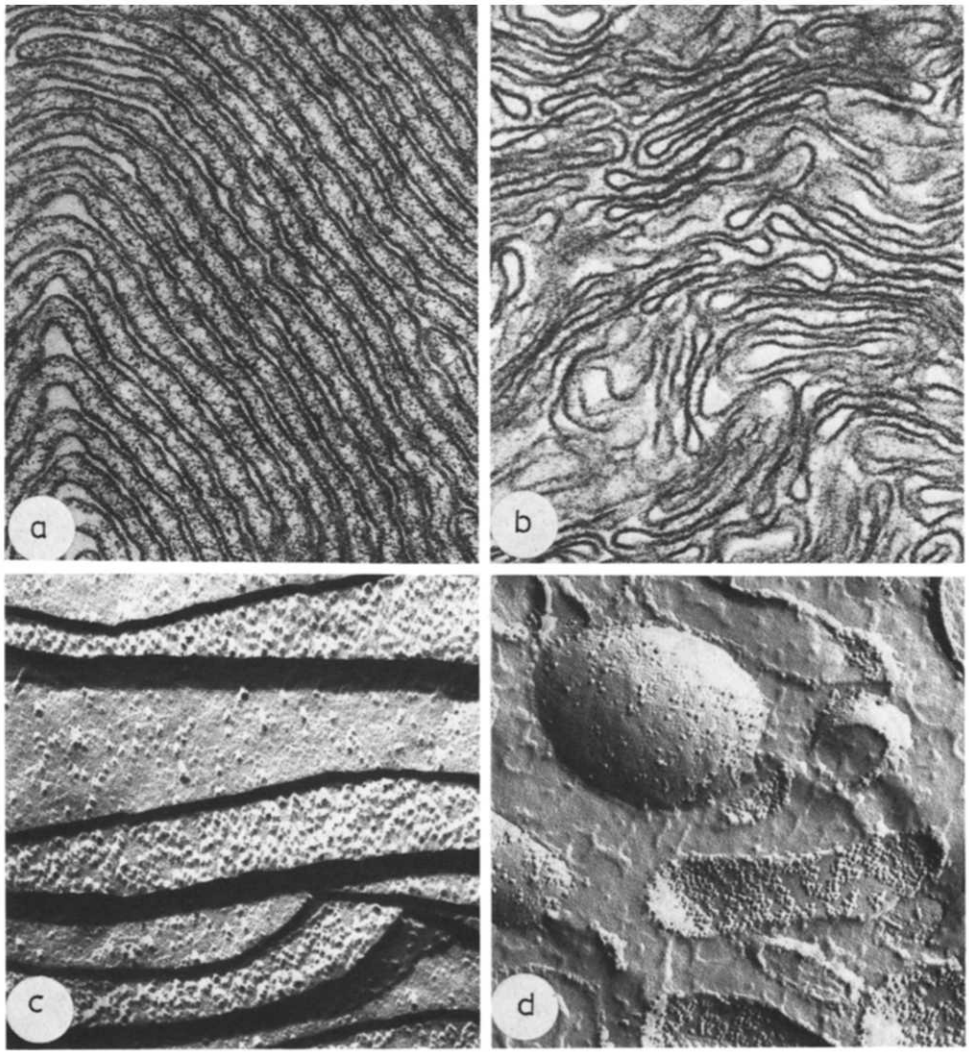


Fig. 2. Electron micrographs of control (a and c) and cholate-extracted (b and d) erythrocyte ghosts centrifuged at high speed (120000 × *g* or 200000 × *g* for 16 h) to form diffraction pellets. (a and b) thin sections and (c and d) freeze-fracture replicas of fast frozen (see text) samples. Samples for freeze-fracture were in 20% glycerol. All micrographs ×72000.

frozen and slow-frozen samples indicates that the slow freezing may induce some aggregation of particles in both P and E fracture faces (Fig. 3d).

X-ray diffraction patterns of frozen (−30°C) samples of untreated membranes and of membranes treated with 0.5% cholate at 4 or 37°C, or with 1.5% cholate at 4°C, showed the first three orders of diffraction relating to a 19.5 ± 0.4 nm periodicity (Fig. 4a). The intensities showed a gradual decrease from 1st to 3rd orders (Fig. 4b). Wide-angle patterns recorded either from frozen fresh discs or from discs that had been frozen during the recording of low-angle patterns and then thawed briefly before re-freezing in the wide-angle camera showed additional broad reflections at 1.6, 0.98 and 0.435–0.44 nm, together with several ice-diffraction rings.

Diffraction patterns from frozen (−30°C) samples of membrane that had been extracted for 30 min with 1.5% cholate were of lower quality than

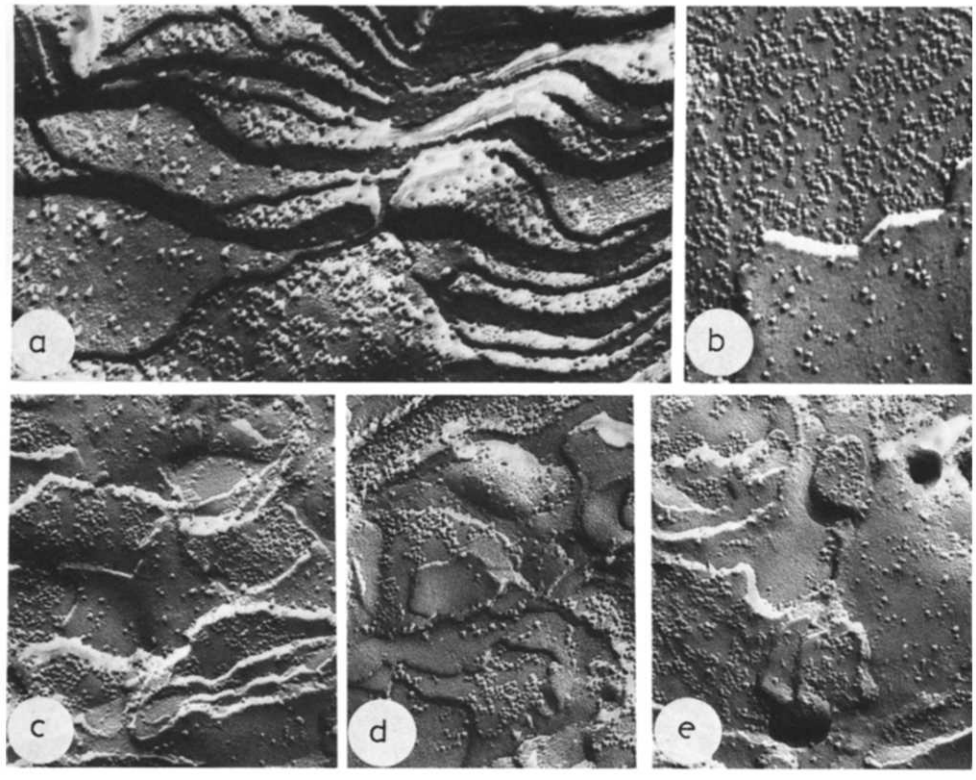


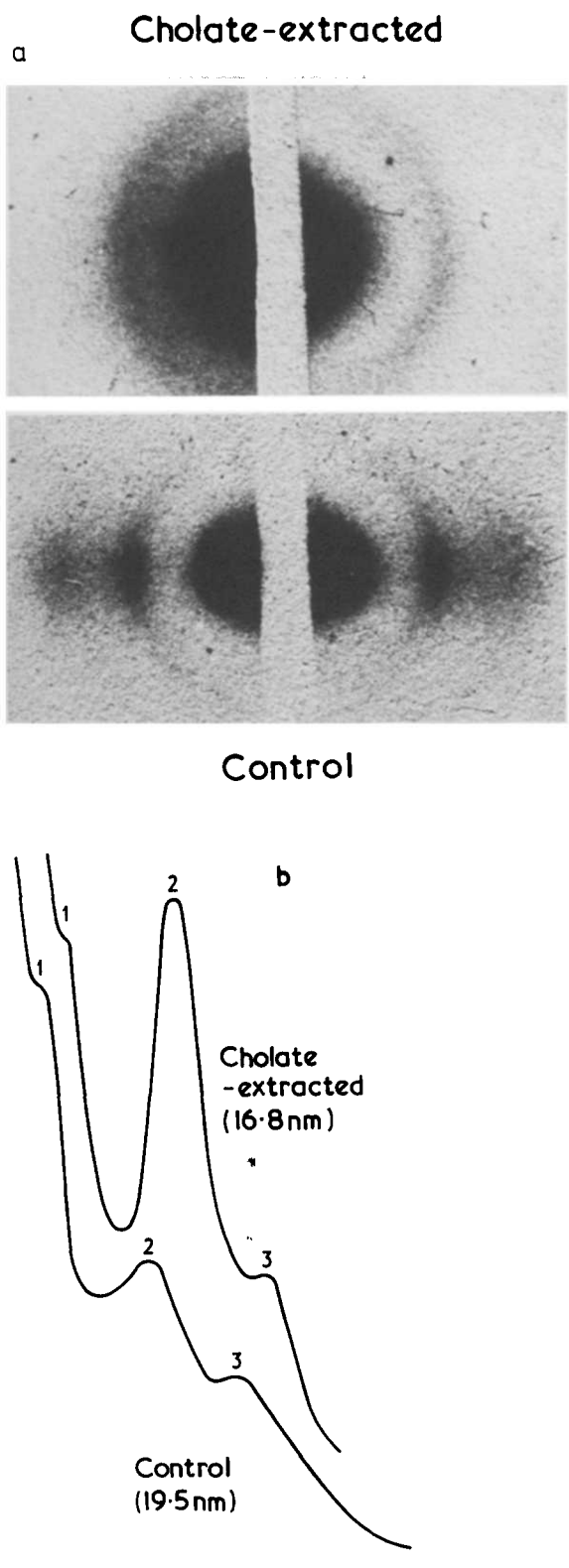
Fig. 3. Electron micrographs of freeze-fracture replicas of control (a and b) and cholate-extracted (c, d, and e) erythrocyte ghosts pre-frozen slowly (18°C min) to −30°C (see text) before rapid freezing to −150°C. Samples were treated with 20% glycerol prior to high-speed centrifugation. All micrographs ×72000.

the controls (Fig. 4a), almost certainly because the membrane vesicles had failed to collapse completely during ultracentrifugation (Fig. 2b) and retained a higher proportion of water in the sample. This would lead, in the frozen state, to a high ice-to-membrane ratio which would be less favourable for diffraction. Nevertheless, such samples showed three orders of diffraction relating to a reduced periodicity of 16.8 ± 0.4 nm (from measurements on second-order diffraction in 12 experiments), and the intensity of the second-order diffraction was greatly enhanced relative to first and third (Fig. 4b). Diffuse rings were still observed at approx. 1 and approx. 0.4 nm and ice-diffraction rings could again be detected on transfer to the wide-angle diffraction camera following long exposures on low-angle cameras. There were no reflections that might be attributable to any additional diffracting phase such as might be expected to arise, for instance, from any major structural reorganisation.

Cholate-treated membranes, to which glycerol had been added before centrifugation and slow or rapid freezing, yielded relatively ill-defined X-ray diffraction patterns, probably because the glycerol restricted the extent of collapse of membrane vesicles during freezing.

Discussion

The levels of protein (40%) and phospholipid (60–70%) removal achieved with 1.5% cholate in these experiments match very closely the findings of our earlier study [3] and the polypeptide analyses confirm that cholate effects a substantial extraction of all polypeptides attributed to the cytoskeletal element of the erythrocyte membrane [3–5].



Vesiculation of the cholate-extracted erythrocyte ghosts is a recognised consequence of the loss of cytoskeletal elements [4]. However, one unfortunate effect of this vesiculation is to make the stacking of membranes by centrifugation much less effective so that measurements of membrane parameters by electron microscopy and by X-ray diffraction become more difficult (see Results for explanation). Nevertheless, the additional condensation of membrane stacks that is brought about by slow freezing does enable effective comparisons to be made by X-ray diffraction and freeze-fracture electron microscopy.

Stepwise fractures through stacks of closely packed collapsed vesicles in the slowly frozen cholate-extracted membranes show a cytoplasmic separation (see Results for definition) that is much reduced as compared with control samples, so that it is now similar to the external separation and the membrane unit therefore more nearly symmetrical. The observation is consistent with the removal of cytoskeletal proteins from the cytoplasmic interface by cholate extraction.

The changes in X-ray diffraction patterns associated with cholate extraction of erythrocyte membranes can also be readily explained on this basis. The observed diffraction intensities cannot readily be converted to accurate electron density profiles of membranes because of uncertainties in intensity measurements, in correction factors and in phasing, but the changes associated with cholate extraction are so pronounced as to make the qualitative implication unmistakable. From electron micrographs, it is clear that the diffraction pattern from the control sample relates to a repeating structure which includes two apposed membrane units, relatively widely separated at the cytoplasmic interface but closely associated at the external interface. Thus, the asymmetry of the two membrane units, implicit in the trend of diffraction intensities in the case of the control sample, probably relates predominantly to the wider separation at the cytoplasmic interface. The domi-

Fig. 4. (a) Low-angle X-ray diffraction patterns from hydrated membrane pellets maintained at −30°C during exposures (16–24 h). Double mirror-(point-)focussing camera. (b) Microdensitometer traces of low-angle X-ray diffraction patterns (one side only). Single mirror-(line-)focussing camera.

nance of the second-order diffraction after cholate extraction would suggest that this source of membrane asymmetry has been removed, as is indeed demonstrated by the electron microscopy and supported by chemical analyses of the extracted and residual proteins. All of the observations are clearly consistent with a loss of protein material from the cytoplasmic interface. The accompanying loss of phospholipid, although extensive, would not be expected substantially to affect the asymmetry of the membrane and therefore should have little effect on the diffraction intensities. In fact, preliminary diffraction studies of frozen samples of phospholipase C-treated membranes from which a substantial proportion of the phospholipid is displaced (as droplets containing the diacylglycerol and ceramide breakdown products [7]), show a very small increase in periodicity and no substantial change in the relative intensity of the second-order diffraction (Finean, J.B. and Hutchinson, A., unpublished data).

The magnitude of the periodicity change (approx. 10%) associated with the loss of asymmetry of the membrane units in the slow-frozen sample is an indication of the extent to which the cytoskeletal material is condensed by the freezing process. Electron micrographs both of thin sections and of freeze-fracture replicas of rapidly frozen membrane stacks and X-ray diffraction studies of control and of glycerol-treated samples [1] indicate that in the fully hydrated membrane preparation, the cytoskeletal layer is many times thicker than the lipid-containing layer, but in the slowly frozen sample, it is condensed to about 10% of the total membrane thickness.

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